"Ameliorative role of *Moringa oleifera* Leaf Extracts Against Acetaminophen and Sitagliptin Induced Toxicity in Swiss Albino Mice."

Submitted

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BY:

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1. Introduction:

The **Liver** and the **Kidneys** are organs that serve essential regulatory roles in most animals. Their primary function is to expel toxins that result from body's metabolism of food and drink.

The **Liver** is the most important and largest glandular organ in the body. It plays an important role in regulating various physiological processes. It is also involved in several vital functions, such as metabolism, secretion and storage. It has great capacity to detoxicate toxic substances and synthesize useful principles (Shanani,1999; Subramoniam and Pushpangadan, 1999).

Since years the Liver has been over-loaded with all sort of harmful synthetic substances, prescribed medicines, recreational drugs, cigarettes , heavy metals and inorganic minerals, The Liver cannot function well once filled with toxic substances. This weakening leads to all kinds of fatal liver diseases and one of the major causes are Drugs.

Drug induced Liver injury (DILI or drug induced hepatotoxicity) is a well recognized problem, DILI accounts up to 10% of all adverse drug reactions. Liver is responsible for concentration, metabolizing and eliminating the majority of drugs that are introduced into the body while some parent drugs ca directly cause hepatotoxicity, it is generally the metabolites of these compounds that lead to DILI. These compounds are processed by a variety of soluble and membrane bound enzymes, especially those related to the hepatocyte endoplasmic reticulum.

Another organ which is prone to toxic metabolites is **Kidney**. The kidneys serve as a natural filter of the body, and remove waste which is expelled through urinary bladder. The main purpose of kidneys is to separate urea, mineral salts, toxins and other waste products from the blood. Kidneys also consume water, salts and electrolytes. At least one kidney must function properly for life to be maintained. In its role as a primary eliminator of exogenous drugs and toxins, the kidneys are vulnerable to develop various forms of injury.

Thus role of these organs in drug metabolism predisposes them to toxic injuries. A person with drug toxicity has accumulated too much of a medication in the blood stream. The effects of the medication are more pronounced at toxic levels, and side effect may be severe. Toxicity may result when the dose is too high, or it may result from prolonged dosage by

which Liver or Kidneys are unable to remove the drug from the blood stream. Many commonly prescribed medications can accumulate in the blood stream and result in toxicity. The drugs which we are going to study for their hepatotoxocity and nephrotoxicity are **Acetaminophen** and **Sitagliptin**.

Acetaminophen (APAP) is a widely used over-the-counter analgesic (pain reliever) and antipyretic (fever reducer). It is commonly used for the relief of headaches and other minor aches and pains and is a major ingredient in numerous cold and flu remedies.

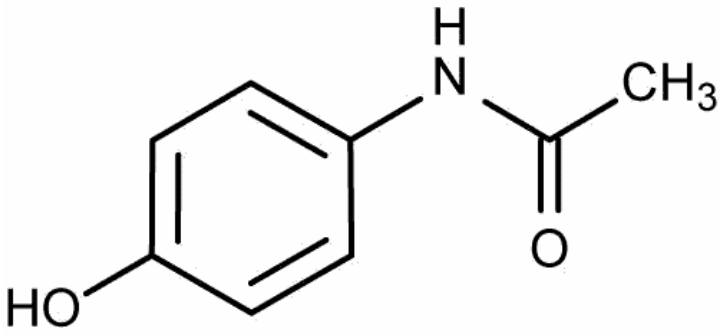


Fig: acetaminophen structure

Acetaminophen is known to cause hepatotoxicity in man and experimental animals when its taken in overdose which leads to elevation of liver enzymes (Kumar *et .al*., 2004; Ahmed and Khater, 2001). It causes severe hepatic necrosis leading to acute liver failure (ALF) after suicidal overdoses (Thomson *et.al*.,1966 ; Black M.,1984 ; Davidson *et.al*.,1986). Unintentional liver injury from self-medication for pain or fever that leads to daily doses exceeding the 4 g/day package recommendations is also well-recognized (Zimmerman *et. al*.,1985; Maddrey *et.al*.,1987; Block *et.al*.,1994; Schiodt *et.al*.,1997). Extended acetaminophen dosing, delay in seeking medical attention, and/or failure to institute NAC therapy are associated with greater morbidity and mortality(Vale *et.al*.,1995 ; Schiodt *et. al*.,1999).

Sitagliptin is an oral hypoglycaemic agent taken in T2DM that enhances the body's own ability to lower the elevated blood glucose and its action is totally glucose dependent. Certain side effects of this drug by over dose or long term use heve been reported. This includes

nausea, arthraglia, myalgia, rash, hives, swelling of the lips, dysphagia, and dyspnea. (Bekur,R. *et.al*., 2010)

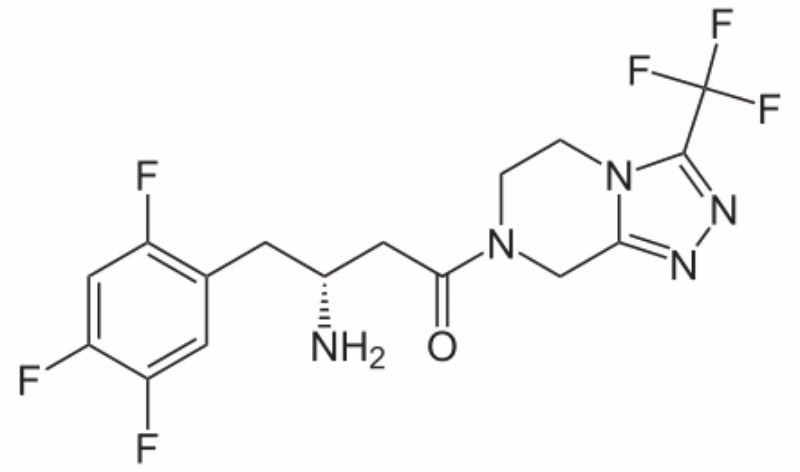


Fig: Sitagliptin structure

In the case of type II diabetes mellitus, the nature of the disorder itself predisposes an increased risk of several organ-specific complications that might be exacerbated by drug treatment. These include drug-induced liver injury associated with a high background rate of hepatitis C infection.

Due to harmful side effects of allopathic drugs, and the fact that these drugs are unavoidable for the patients who are on their medication, there has been a continuous research for a natural/herbal safer agent that can be taken along with the drugs so that it can prevent/cure the harmful effects.

Plant products as medicines

The use of natural remedies for the treatment of liver and kidney diseases has a long history and medicinal plants and their derivatives are still used all over the world in one form or the other for this purpose.

Ayurveda with its vast modalities is an integral spiritual science, which is gifted to the universe from the ancient enlightened Vedic culture. This science of life, which is emerging

as the global medicine accords a high place for the dark green leafy vegetables in the daily diet. India, with its enormous natural resources, is bestowed with thousands of varieties of leafy vegetables, which is highly energy giving. India is the second largest producer of fresh vegetables(Sathianaraynan *et .al*, 2009).

From time immortal, man depended on plants as medicine. These remedies are most efficacious as they are taken directly from nature. They are amazingly nutritious and powerhouse of antioxidants.

Moringa oleifera is a perennial softwood tree with timber of low quality, but which for centuries has been advocated for nutritional, medicinal and industrial uses. *Moringa oleifera* belongs to the family moringaceae. The leaves of *M.oleifera* are rich sources of beta carotene, vitamin C, protein, iron and potassium. They are highly nutritious. The alcoholic extract of these drumstick leaves have radical scavenging capacities and antioxidant activities. (Siddhuraju *et.al.*2003). The therapeutic effects of *M. oleifera* include: antibiotic (Fahey *et. al*., 2002; Haristoy *et .al.,* 2005), anticancer (Guevara *et. al.,* 1999: Bharali *et .al.,* 2003), antiulcerogenic effects (Akhtar and Ahmad, 1995), analgesic (Rao and Ojha, 2003), antiurolithiatic (Bennett *et .al*., 2003) and larvicidal activities (Sharma *et. al.,* 2006).

2. Review of literature:

Primary function of liver and kidneys is to expel toxins that results from body's metabolism of food and drink. Toxins include end products, micro organisms, pollutants, drugs and alcohol. These metabolisms are generally formed by the overdose or long term use of some allopathic drugs. The liver with its multiple cell types and numerous functions can respond in many different ways to acute and chronic insults (Bodakhe and Ram, 2007).

Fortunately, the kidneys are also equipped with a variety of detoxification mechanisms and have considerable functional reserve and regenerative capacities. A slight alteration in the structure and function may result in portal hypertension, ascites, and jaundice and increased bleeding, tubular epithelial cell necrosis in both proximal and distal parts of the tubules (Bjorck *et. al.,* 1988) and cause multiple metabolic changes affecting other organs as well. Liver and kidney related complications are some of the fatal disease in the world today.

The presence of chronic liver diseases may increase the hepatotoxic potential of some drugs, such as non steroidal anti inflammatory drugs (NSAIDS) according to Society of General

Internal Medicine (Riley and Smith, 1999). Acetaminophen is a kind of NSAID that helps in relieve pain and reduce fever. High doses of acetaminophen can produce liver damage. According to the American Academy of family physicians NSAIDS are also linked to acute renal failure.They induce acute renal failure by inhibition of the production of prostaglandins, a hormone like substance, resulting in poor renal perfusion (Malay and Richard, 2000).

Acetaminophen toxicity is one of the major causes of poisoning worldwide (Gunnell *et.al*., 2000), and its overdose is commonly associated with irreversible hepatic (Nelson, 1995) and renal damages (Placke *et .al*., 1987) where glomerular filteration rate decreases over days to weeks and excretion of nitrogenous waste is reduced So, increase in blood urea nitrogen (BUN) and serum creatinin level is observed. The toxicity is mediated by the activity of its reactive metabolite known as *N*-acetyl-*p*-benzoquinoneimine (NAPQI) by cytochrome P450 that covalently binds to protein (Mitchell *et.al.*, 1973), which is detoxified by intracellular glutathione (GSH) (Borne, 1995). Therefore, an overdose of the drug will saturate the conjugation pathways of GSH and cause depletion of cellular GSH.

Another drug which is specifically used by type II Diabetes patients is Sitagliptin. **Sitagliptin** is an oral hypoglycaemic agent that enhances the body's own ability to lower the elevated blood glucose and its action is totally glucose dependent. Sitagliptin inhibits DPP-4 enzyme induced inactivation and degradation of incretin hormones. The enhanced incretin action stimulates insulin release and decreases glucagon secretion, thereby lowering haemoglobin A1c and fasting and postprandial glucose levels (Iltz *et.al.* 2006). In the case of type II diabetes mellitus, the nature of the disorder itself predisposes an increased risk of several organ-specific complications that might be exacerbated by drug treatment. These include drug-induced liver and kidney injury associated with a high background rate of hepatitis C infection (Chitturi *et.al*., 2002).

From time immortal, man depended on plants as medicine. Nature abounds in a variety of green leaves, and the researches shows that this provides the ready answers to the ordinary ailments of life. Previous research has shown medicinal plants with nephroprotective properties to mediate their protection by their antioxidant and/or free radical scavanging activities due to high concentration of flavonoids and alkaloids they contain (Miller and Rice-Evans, 1997; Adneye and Benebo, 2008).

Moringa oleifera is a perennial softwood tree with timber of low quality, but which for centuries has been advocated for nutritional, medicinal and industrial uses. *Moringa oleifera*

belongs to the family Moringaceae. The hepatoprotective effect of ethanolic extract of *Moringa oleifera* on liver damage caused by antitubercular drugs was evaluated. Hepatoprotective effect of *Moringa oleifera* against APAP is evident by the restoration of Alanine Aminotransferase (ALT), Aspertate Aminotransferase(AST) and Alkaline phosphatase (ALP). Concurrently, significant preservation of liver histology was observed in the groups that were pretreated with *Moringa oleifera* leaf extract (Fakurazi *et.al.,* 2008). In terms of growth parameters the fresh and dehydrated drumstick leaves were found to be a better source of vitamin A than synthetic vitamin A. The ethyl acetate extract of dried leaves was found to have significant wound healing property (Hukkeri *et.al.*,2006). At lower concentration it also regulates hyperthyroidism. They cause the reduction of serum T_3 concentration but increase the serum T_4 by inhibiting the peripheral conversion of T_4 to T_3 (Tahiliani *et.al.*,2000). The leaves of *Moringa oleifera* have proved beneficial in the treatment of gastric and duodenal ulcers.

Powder from seed kernels of *Moringa* works as a natural coagulant and is used in the rural areas to clarify very turbid water (Gassenschmidt *et.al.,* 1995). Immature seeds of *M. oleifera* are used in recipes, the leaves are extensively used as vegetable in many parts of the world and the roots can be made into a condiment similar to horseradish (Prajapati *et al*., 2003). In addition, its beneficial roles in human immunodeficiency/acquired immune deficiency disease (HIV/AIDS) (Burger *et al.,* 2002) and effects on regulation of thyroid hormone status in adult male and female rats have been reported (Pankaj and Anand, 2000).

3. Objectives of study:

- To evaluate the hepatotoxicity and nephrotoxicity of two commonly used drugs: Acetaminophen and Sitagliptin.
- To screen the plant (*M. oleifera*) for its phytochemical properties and presence of bioactive compounds.
- To assess the hepatoprotective and nephroprotective role of crude extract and bioactive compounds against damage caused by the chronic exposure of drugs in liver and kidney of Swiss albino mice.

4. Methodology and plan of work:

In this proposed plan hepatoprotective and nephroprotective role of *Moringa oleifera* (drumstick leaves) will be studied in Swiss Albino mice chronically exposed to two allopathic drugs- Acetaminophen and Sitagliptin. Acetaminophen (Paracetamol N-Acetyl p-aminophen) is an effective analgesic-antipyretic drug which is often used to treat pain and fever. The most serious adverse effect of acute overdose of acetaminophen is dose-dependent, potentially fatal hepatic necrosis (Thomas, 1993) which may be associated with renal tubular necrosis (Goodman and Gilman,1996). The number of self poisoning suicides with acetaminophen has grown alarming in recent years (Simkin *et.al*., 2012). It is available without prescription in many parts of the world (Goodman and Gilman, 1996). Another drug used for the study is Sitagliptin which is well-tolerated, moderately efficacious, weight neutral oral anti-diabetic drug, with a low incidence of hypoglycaemia. The ever increasing burden of Type-II Diabetes mellitus (T2DM) and inadequate control in the majority of patients has led to a quest for newer therapeutic options. Patient has to take these drugs for very long period, sometimes throughout their life. Exposure to these drugs for a longer period affects the patient liver and kidney adversely (Subbarayan and Kipnes, 2011).

A. PREPARATION OF EXTRACT:

1. ***Moringa oleifera*:** leaves will be selected for study. The leaves will be shade dried for 3 weeks. The dried leaves then grounded into powder form and can be at 4°C until further use. The leaves powder then will be extracted using 80% hydro alcoholic solvent (80% ethanol: 20% distilled water) with constant shaking at room temperature for overnight. The extract will be filtered and the residue can then be resuspended in ethanol for 48 hours and refiltered. The filtrate will then concentrated using a rotary evaporator under reduced pressure at 40 °C and then lyophilized using a freeze dryer. A dark green mass was obtained and stored at -20°C until further use. The crude extract, will be resuspended in distilled water before administration to the animals (Hossain et al., 1992).

B. PHYTOCHEMICAL ANALYSIS:

1. Isolation and purification of bioactive compound will be done using TLC and column chromatography (Sawhney and Singh, 2009).

2. Quantitative estimation of these selective bioactive compounds of the above plant will be done by HPLC method (Sakakibara *et.al*., 2003; Ndong *et.al*., 2007).

Experimental protocol

Maintenance of Animals: Both sexes of Swiss Albino mice 6-8 weeks old will be maintained in standard condition: 23±2°C.

Relative Humidity: 55±10%.12:12 h LD Cycle and allowed free access to food and water. They will be housed in separate polypropylene cages containing sterile paddy husk as bedding material.

Group I: Hepatotoxic and Nephrotoxic study of drugs

Hepatotoxiciy of selected drugs Acetaminophen and Sitagliptin will be studied at three different dose levels. Doses will be computed on the basis of prescribed doses to human. The average recommended human dose will be converted to that of mice by a standard conversion table of Ghosh, 2005.

Depending on the dose animals will be exposed to drugs by oral gavage for 7, 14 and 30 days.

Group II: Determination of dose related Hepatoprotective and Nephroprotective role of plant

Animals will be provided with crude extract of the selected plant at three dose levels from10 days prior to and during the subchronic administration (for 10 days) of drugs along with different dose levels. Animal will be provided with crude extract for another 10 days after the seizure of drug administration.

Autopsy interval: After 10, 20 and 30 days of exposure to the plant extract treatment.

Group III: Hepatoprotective and Nephroprotective role of plant bioactive compound

Above experiment (Group II) will be repeated taking selected **bioactive compounds** of plants in place of crude extract.

Group IV: Study on the effects of plant extract (crude) and selected bioactive compounds on the liver functions

Animals will be provided with crude extract and bioactive compounds at low dose levels for two months along with the drug dose. Autopsy intervals: 7, 15, 30 and 60 days.

Controls will be run simultaneously with each experimental group. The mice will be monitored for change in body weight and food consumption during the experiment.

Parameters:

HISTOLOPATHOLOGICAL STUDY

The liver tissue will be dissected out and fixed in 10% formalin, dehydrated in gradual ethanol (50–100%), cleared in xylene and embedded in paraffin wax. Then, it will be cut into 5 µm sections and stained with haematoxylin–eosin dye for photomicroscopic observations of hepatocytes of treated groups through light microscopy. The parameters for the study will be fatty and hydrophobic changes in hepatocytes, deformation in hepatocytes, focal necrosis, congestion in central vein or sinusoids and bleeding area in hepatic lobes. The parameters for the study of renal structure will be moderate hydropic glomerular (**GLM**) and tubular degenerations (**TD**) obliterated proximal convoluted tubular lumen (**OPC**) and obliterated distal convoluted tubular lumen (**ODC**).

BIOCHEMICAL STUDIES

Animals will be anesthesized with ether. Blood samples will be collected from jugular vein. The blood sample will be allowed to clot for 45 min at room temperature. Serum will be separated by centrifugation at 600X g (15 min) and analyzed for the following:

Serum Analysis

- **Aspertate Aminotransferase(AST)**
- **Alanine Aminotransferase(ALT)**
- **Alkaline phosphatase(ALP)**
- **Serum Acid Phophatase(ACP)**
- **Gamma Glutamyl Transpeptidase(GGTP)**
- **Total Bilirubin**
- **Total Proteins**
- **Total Albumin**

(Using diagnostic kits)

After the collection of blood, the liver and kidney will be immediately excised, washed with cold saline, blotted and a part of them will be minced and a part of it will be minced and homogenized for

- **Superoxide Dismutase (SOD)** (Marklund and Marklund, 1974)
- **Catalase(CAT)** (Aebi,1984)
- **Reduced Glutathione(GSH)** (Moron *et.al.,*1979)

- **Glutathione Peroxidase(GP_X)**(Paglia and Velentine,1967)
- **Glutathione S transferase(GST)**(Habig *et.al.,*1974)
- **Lipid Peroxidation(LPO)** (Ohkawa *et.al.,*1979)

Then, a liver microsomal fraction will be prepared (Schneider and Hogeboom 1950) and the cytochrome P450 enzyme content in this fraction will be measured from a reduced carbon monoxide difference spectrum (Omura and Sato, 1964), respectively.

Hepatoprotective Activity (%H): The hepatoprotective activity will be calculated as follows:

1-(T-V)/(C-V) X 100

Where T= mean value of the drug and plant treatment group

C= mean value of the drug treatment group only

V= mean value of the normal control animal

Assessment of serum uric acid, urea and creatinine

Blood samples, obtained directly from the heart chamber of the anaesthetized rats kept at the temperature of 4 ºC for 6 hours before they will be centrifuged using Laboratory Centrifuge at 3000 rpm at same temperature for 20 minutes in order to separate the sera.

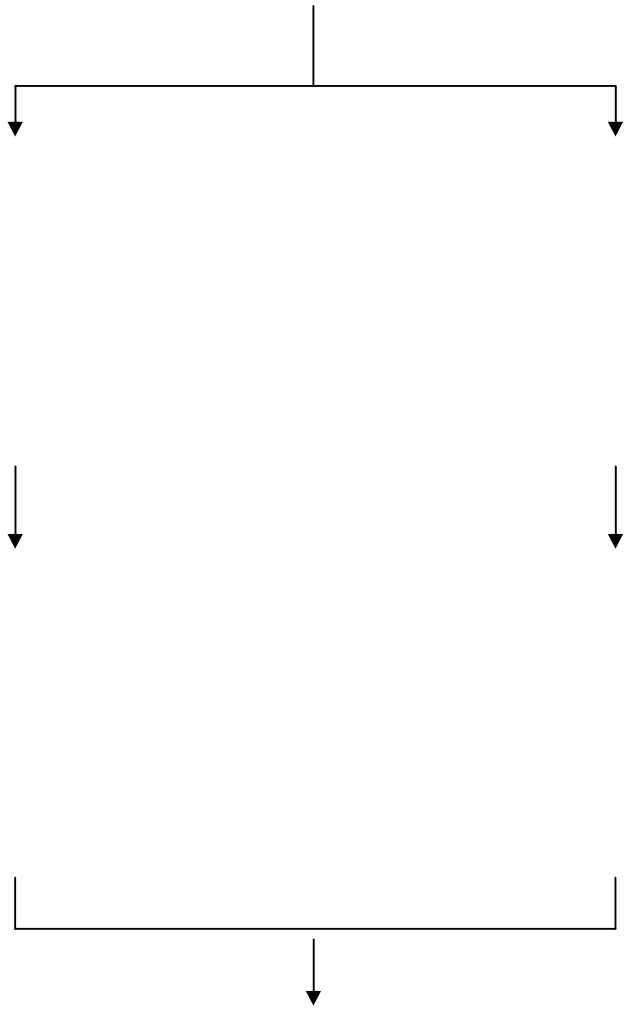
Serum Analysis

- **urea and creatinine** levels (modified methods by Marsh *et.al.*, 1965 and Biod and Sirota, 1948) using standard diagnostic test kits
- **proteins** (Plummer, 2001) and activities of enzymes such as
- **LDH** (Abell *et.al.* 1952)
- **ASAT and ALAT** (King 1965)
- **ACP and AKP** (Yatzidis 1960)
- **Na+1-K+1 ATPase, Ca+2 ATPase and Mg+2 ATPase** (Bergemeyer and Bernt, 1963)

Statistical Analysis:

All the experiments will be carried out in triplicates and the data will be expressed as Mean ± S.D. One way Anova will be calculated to determin the P value of each group.

Fig 1: Hepatotoxic and Nephrotoxic study of drugs (Group I



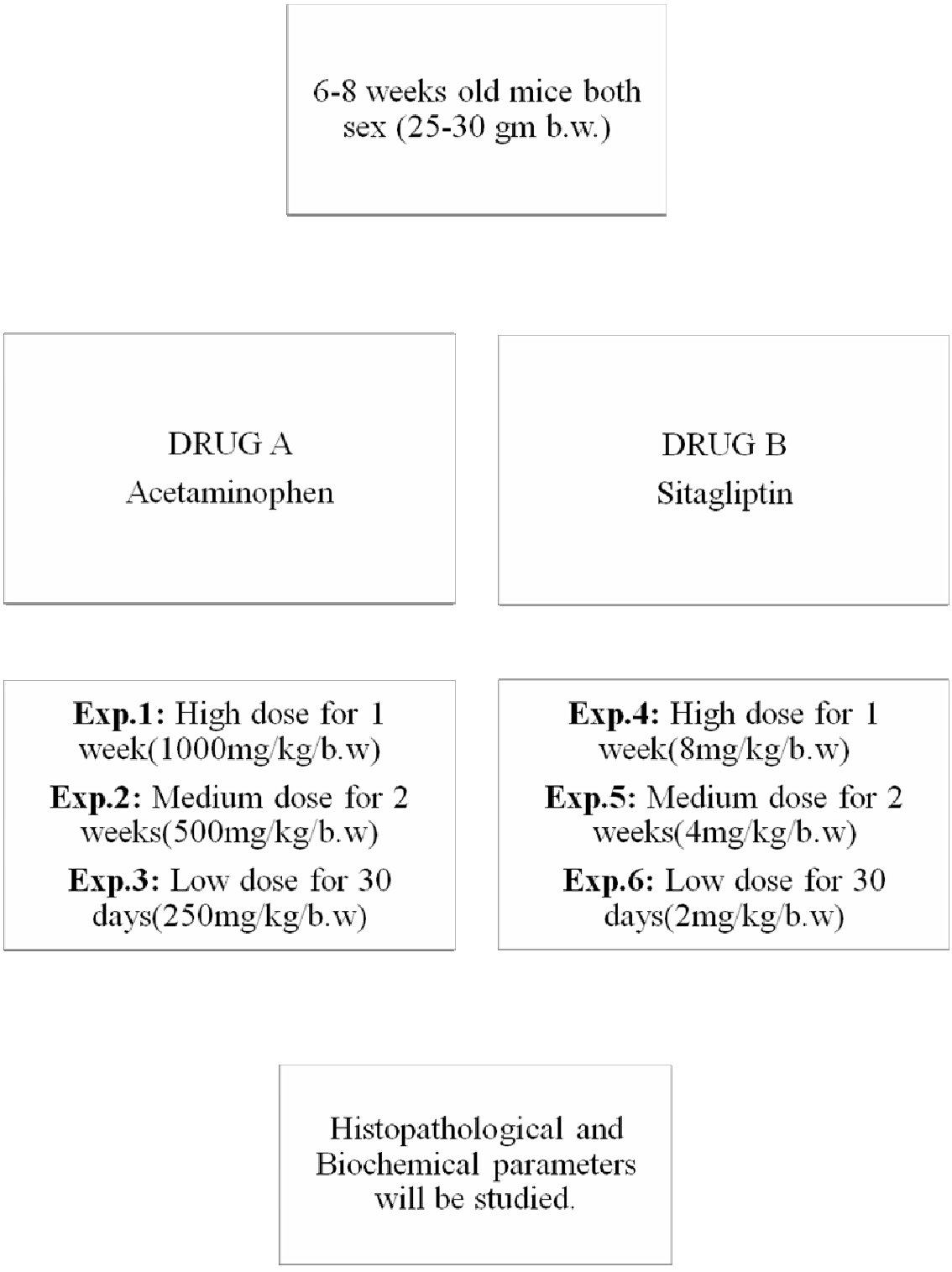

6-8 weeks old mice both sex (25-30 gm b.w.)
DRUG A
Acetaminophen
DRUG B
Sitagliptin
Exp.1: High dose for 1 week(1000mg/kg/b.w)
Exp.2: Medium dose for 2 weeks(500mg/kg/b.w)
Exp.3: Low dose for 30 days(250mg/kg/b.w)
Exp.4: High dose for 1 week(8mg/kg/b.w)
Exp.5: Medium dose for 2 weeks(4mg/kg/b.w)
Exp.6: Low dose for 30 days(2mg/kg/b.w)
Histopathological and Biochemical parameters will be studied.

Fig. 2: Study of ameliorative role of plant extract against chronic exposure of each drug and on liver and kidney (Group II and III)

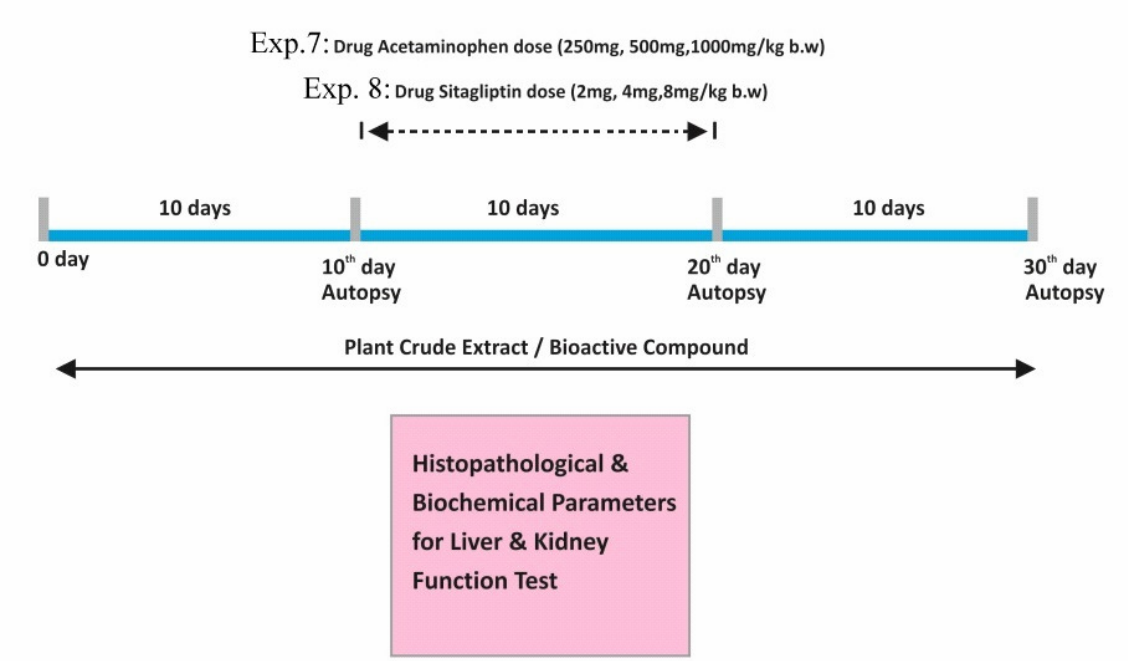
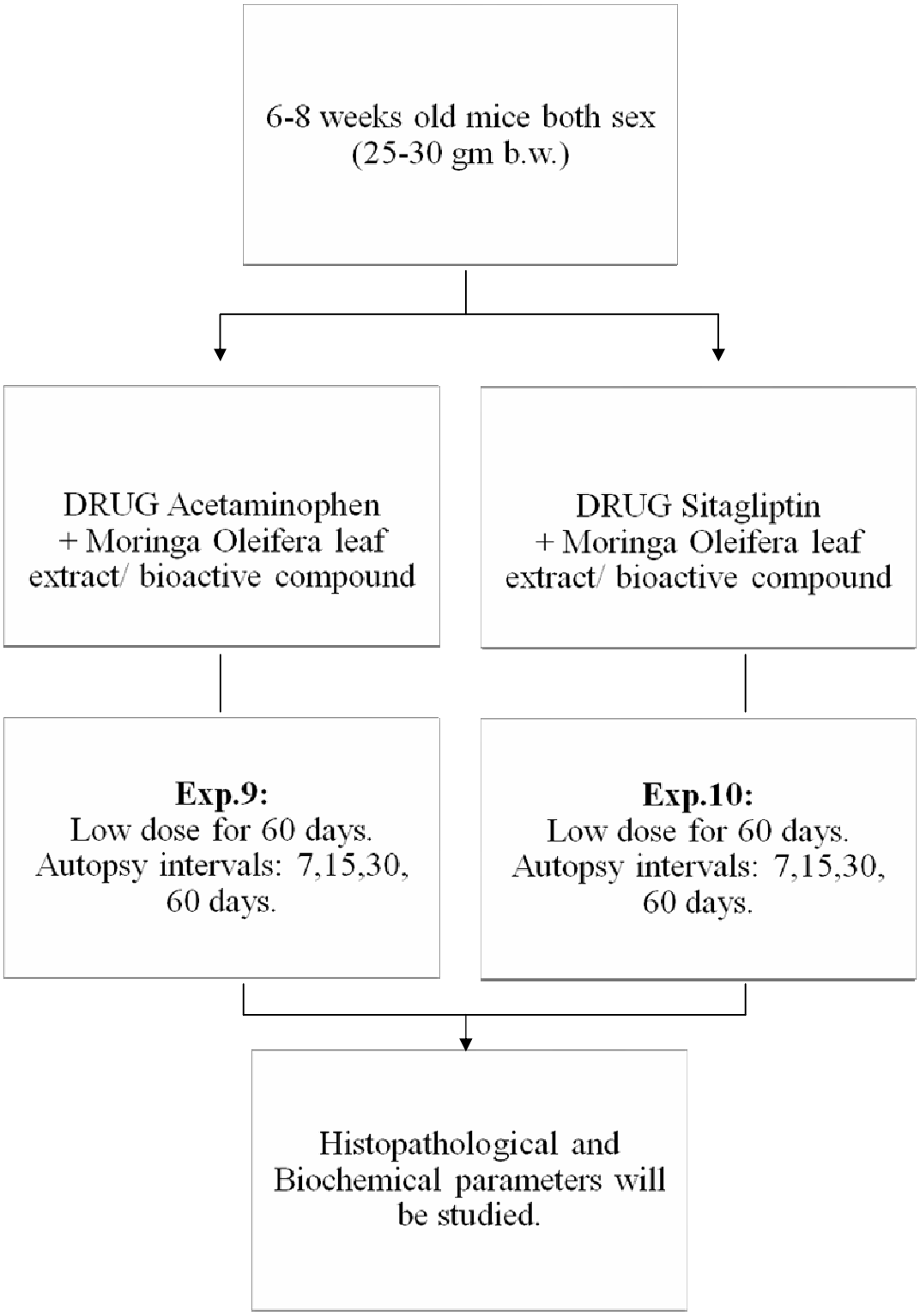

Exp.7: Drug Acetaminophen dose (250mg, 500mg,1000mg/kg b.w)
Exp. 8: Drug Sitagliptin dose (2mg, 4mg,8mg/kg b.w)
10 days
10 days
10 days
0 day
10th day Autopsy
20th day Autopsy
30th day Autopsy
Plant Crude Extract / Bioactive Compound
Histopathological & Biochemical Parameters for Liver & Kidney Function Test

Fig.3: Study on the effects of plant extract (crude) and selected bioactive compounds on the liver and kidney functions (Group IV)



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